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Research Article

Evaluation of *MexB* and *MexY* Genes Expression in Carbapenem-Resistant *Pseudomonas aeruginosa* Strains Isolated from Educational Hospitals of Qazvin and Tehran, Iran

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Abstract

Background: *Pseudomonas aeruginosa* is one of the most common Gram-negative pathogen causing nosocomial infection worldwide. The emergence of multi-drug resistant strains has become a major clinical concern in recent years. Resistance-nodulation-cell division (RND) family efflux pumps play an important role in the development of multidrug resistance in *P. aeruginosa*.
Objectives: The aims of the present study were to evaluate the antimicrobial susceptibility against carbapenems used and to evaluate the gene expression of two resistance nodulation cell division-type efflux pump systems (*MexB* and *MexY*) among carbapenem non-susceptible *P. aeruginosa*.
Methods: A total of 256 non-repetitive *P. aeruginosa* were collected from the different clinical samples of patients admitted in Qazvin and Tehran educational hospitals. Antimicrobial susceptibility testing was performed by standard Kirby-Bauer method. The expression of *MexB* and *MexY* genes was assessed by quantitative real-time polymerase chain reaction (qRT-PCR).
Results: In total, 107 (41.8%) isolates were non-susceptible to imipenem and/or meropenem. All carbapenem non-susceptible isolates were positive for the presence of *MexB* and *MexY* genes. The expression of *MexB* (P value = 0.004) and *MexY* (P value = 0.001) genes was significantly increased, compared to standard carbapenem sensitive strains.
Conclusions: The findings of the present study showed the considerable presence of carbapenem resistance among the clinical isolates of *P. aeruginosa*. The overexpression of *MexB* and *MexY* genes is also established among these resistant isolates collected from studied hospitals in Iran.

Keywords: Carbapenem, *MexB*, *MexY*, *Pseudomonas aeruginosa*

1. Background

Pseudomonas aeruginosa is an opportunistic pathogen that has been known as one of the significant pathogen contributing in nosocomial infection worldwide (1). This organism is able to cause severe infection in patients, most notably those with cystic fibrosis or hospitalized in intensive care units (2, 3). The extensive use of broad-spectrum antibiotics contributes to the emergence of multidrug resistant *P. aeruginosa* (MDRPA). MDR in *P. aeruginosa* is defined as the resistance to three or four of antibiotic classes including β-lactams, aminoglycosides, and fluoroquinolones (4). Carbapenems such as meropenem and imipenem are classified as β-lactam antibiotics that are routinely used as therapy for *P. aeruginosa* (5). These antibiotics have a broad spectrum of activity but in recent years, the development of carbapenems resistance has been showed among *P. aeruginosa* isolates (6).

P. aeruginosa is resistant to carbapenem through several mechanisms: first, diminished cell-wall permeability; second, the constitutive expression of various efflux pumps; and third, the production of antibiotic-inactivating-hydrolyzing enzymes (7).

Bacterial antimicrobial efflux transporters have generally been classified according to amino acid sequence homology. Five superfamilies have been identified including the major facilitator superfamily (MFS) (8), the ATP-binding cassette family (9), the resistance-nodulation-division (RND) family (10, 11), the small multidrug resistance (SMR) protein family (12), and multidrug and toxic compound extrusion (MATE) family (13).

RND family is the most common multidrug efflux systems in clinical Gram-negative bacteria especially in *P. aeruginosa* (10). Several RND-type multidrug efflux systems in *P. aeruginosa* have been identified, among those MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM are reported to be significant determinants among multidrug resistant clinical isolates (14). MexAB-OprM and MexXY-OprM pumps show natural resistance against antimicrobial and disinfectant compounds. MexAB-OprM system provides resistance to a broad spectrum of antibiotics including tetracycline, chloramphenicol, quinolones, trimethoprim, and most β-lactams and MexXY-OprM contributes to the low intrinsic susceptibility to aminoglycosides, tetracycline, and erythromycin (15).

The aims of this study were to determine the antimicrobial susceptibility against carbapenem among *P. aeruginosa* isolated from patients in hospitals of Tehran and Qazvin provinces, and also to evaluate the existence and expression of *MexB* and *MexY* genes as important factors of drug resistance among these isolates.

2. Methods

2.1. Study Design and Identification

A total of 256 non-repetitive *P. aeruginosa* isolates were collected from the various clinical samples of patients admitted in educational hospitals of Qazvin and Tehran. These bacterial isolates were collected from February, 2012 until December, 2014. The study was approved by the ethics committee of Qazvin University of Medical Sciences (code IR.QUMS.REC.1394.147). The isolate identification was done by standard laboratory methods including bacteriologic and biochemical tests such as Gram stain, oxidase test, growth on cetrimide agar medium (Liofilchem, Italy), the ability to grow at 42°C, O/F (Oxidation-Fermentation) test, and pigment production. The bacterial isolates were obtained from different clinical specimens including urine, sputum, wound, tracheal aspirate, blood, and bronco alveolar lavage.

2.2. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed by Kirby-Bauer disc diffusion method against imipenem (10 μg), meropenem (10 μg) (Mast Diagnostics Group Ltd, Merseyside, UK) according to the CLSI guideline (16). For quality control in antimicrobial susceptibility testing, *P. aeruginosa* ATCC 27853 was used.

2.3. Detection of Mexy and Mexb Genes by PCR Assay

PCR assay was performed for carbapenem non-susceptible *P. aeruginosa* isolates for the detection of *MexY* and *MexB* genes using specific primers (Table 1). DNA extraction was performed using a commercial kit (Bioneer, South Korea) according to the manufacturer's instruction. PCR amplifications were performed in a thermocycler (Applied Biosystems, USA) as follows: 7 minute at 95°C and 35 cycles of 1 minute at 95°C, 1 minute at specific annealing temperature and 1 minute at 72°C, followed by a final extension at 72°C for 10 minute. Amplification reactions were prepared in a total volume of 25 μL (24 μL of PCR master mix plus 1 μL of template DNA), 10 mM dNTP mix at a final concentration of 0.2 mM, 2.0 U of Taq DNA polymerase, 50 mM $MgCl_2$ at a final concentration of 1.5 mM, 1 μM of each primer, and 1X PCR buffer. PCR products were visualized on ethidium-stained 1% agarose gels in gel documentation system (UVtec, UK).

Table 1. Primers Used in This Study

Genes	Sequence (5' → 3')	Size, bp	References
MexY	F: CCGCTACAACG-GCTATCCCT	246	(17)
	R: AGCGGGATC-GACCAGCTTTC		
MexB	F: GTGTTCG-GCTCGCAGTACTC	244	(17)
	R: AAC-CGTCGGGATTGAC-CTTG		

2.4. RNA Extraction and Synthesis of cDNA

For the extraction of total RNA, *P. aeruginosa* isolates were cultured in 1.5 mL LB broth, left in a shaker incubator (180 rpm) at 37°C for 18 - 24 hours. Plates were incubated overnight at 37°C and then were centrifuged (8,000 rpm) at 4°C for 7 minutes. For the extraction of total RNA, the sediment was used using a commercial kit (RNeasy kit, Qiagen, Germany), according to the manufacturer's protocol.

To eliminate the genomic DNA, a commercial RNase-free DNase kit (DNase, RNase-free, Fermentas, Thermo Scientific, USA) was used according to the manufacturer's protocol. To prepare cDNA, 5 μL of extracted RNA was added to 15 μL deionized water and converted to cDNA in a total volume of 20 μL using a commercial kit (cDNA kit, BioNeer Pacific, Australia) according to the manufacturer's protocol. Electrophoresis on agarose gel was performed for quality and quantity of extracted RNA. In order to determine *MexY* and *MexB* gene expression, real-time PCR was performed using a commercial kit (SYBR® Green2X Master Mix; Applied Biosystems, USA) on an Applied Biosystems 7,500 real-time PCR (Fort Collins, Colorado, USA). Briefly, 0.5 μL of each primer, 5 μL cDNA, 4 μL deionized water, and 10 μL Master Mix were added to a standard 96-well microplate (Applied Biosystems; USA). PCR reactions were performed in final volume of 20 μL for 45 cycles. Melting curve was drawn to evaluate the specificity of PCR reaction, followed by electrophoresis of PCR products on 2% agarose gel. The specific primers to evaluate the changes of *MexY* and *MexB* genes expression in carbapenem non-susceptible *P. aeruginosa* isolates are shown in Table 1 (17). For normalization of target gene expression levels, the housekeeping rpoD gene was used as internal standard control and the standard *P.*

aeruginosa strain (ATCC 27853) for comparative evaluation of the *MexY* and *MexB* genes.

2.5. Statistical Analysis

One-Sample Student's t-test was used to compare the mean values of quantitative variables; comparison of *MexY* and *MexB* genes expression obtained for carbapenem non-susceptible isolates with those of obtained for standard (control) *P. aeruginosa* PAO1 (ATCC 27853) strain.

3. Results

The results of antimicrobial susceptibility testing indicated that 13 (5.1%) and 3 (1.2%) isolates were non-susceptible to meropenem and imipenem, respectively. Ninety-one (35.5%) isolates were non-susceptible to imipenem and meropenem, simultaneously. In total, 107 (41.8%) isolates were non-susceptible to imipenem and/or meropenem. PCR assay showed that all carbapenem non-susceptible isolates were positive for the presence of *MexY* and *MexB* genes. In total, qRT-PCR showed different expressions in different clinical isolates. The expression of *MexB* (P value = 0.004) and *MexY* (P value = 0.001) genes was significantly increased, compared to standard carbapenem sensitive strains.

4. Discussion

Resistance to multiple antibiotics in *P. aeruginosa* is one of the most serious therapeutic challenges for treatment of both community-acquired and nosocomial infections (1). Treatment of MDRPA is difficult due to limited choice of antibiotics because this bacteria is capable of showing resistance to antibiotics by intrinsic and acquired resistance ways (4). The constitutive expression of efflux pumps such as MexAB-OprM and MexXY-OprM have an important role in resistance to several classes of antibiotics (14). Carbapenems such as imipenem and meropenem are frequently used as the last therapeutic strategy against the infections caused by MDRPA. However, the emergence of carbapenem resistant isolates has become a serious clinical concern around the world (6).

In the present study, resistance to imipenem and/or meropenem was 41.8% which is higher than the results of previous studies have been reported by Shahcheraghi et al. (12.4%) and Japoni et al. (30%) in Iran (18, 19), but lower than that found in two studies carried out by Sepehriseresht et al. (56%) and Khosravi et al. (41%) in burn patients in Iran (20, 21). It seems, the variable susceptibility rate of carbapenems among *P. aeruginosa* isolates in different studies could be due to varied antibiotic usage profiles in different geographic regions.

In the present study, all isolates were positive for the presence of *MexY* and *MexB* genes; the data showed the presence of these efflux pumps are common in *P. aeruginosa*. Our study also showed that overexpression of *MexB* and *MexY* genes is one of the important mechanisms to resist against carbapenems. In a similar study from Iran, Arabestani et al. showed that all clinical isolates of *P. aeruginosa* were positive for the presence of MexAB-OprM genes but a different expression of MexR, OprD, and *MexB* genes was observed in resistant isolates (22). Masuda and coworkers showed that MexXY contributes to the intrinsic resistance to antibacterial drugs (23). Dumas et al. reported that MexX and *MexY* genes were induced 8 to 12 times in the presence of tetracycline and mexC/oprJ and mexE/oprN gene expression levels were increased 30- to 250-fold and 100- to 760-fold in mutant isolates, respectively (24). Hocquet et al. in two French teaching hospitals showed the major role of MexXY-OprM in the development of cefepime resistance among clinical strains of *P. aeruginosa* (25). Terzi and coworkers in Turkey showed that *MexB* was overexpressed in 15 of 22 and *MexY* in 19 of 22 of multidrug resistant *P. aeruginosa* isolates. They, also, reported that transcription of *MexB* was directly correlated with meropenem resistance in the majority of their isolates (26).

4.1. Conclusions

Findings of the present study showed the considerable presence of carbapenem resistance among the clinical isolates of *P. aeruginosa*. Overexpression of *MexB* and *MexY* genes is also established among these resistant isolates which highlights the need for establishing suitable strategies to effectively treat patients and prevent the further distribution of these resistant organisms in our hospitals.

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Footnotes

Authors' Contribution: Conception and design of the study, Amir Peymani; laboratory work, Amir Peymani, Hadi Rahimi, Shahin Bolori, and Taghi Naserpour Farivar; data analysis and interpretation, Taghi Naserpour Farivar.

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